THE PERIOD PREVALENCE OF DOUBLE EXPRESSION DIFFUSE LARGE B-CELL LYMPHOMA PATIENTS AND ITS ASSOCIATED RISK FACTORS AND TREATMENT RESPONSE IN HOSPITAL UNIVERSITI SAINS MALAYSIA FROM YEAR 2004 TO 2015

DR TEH CHEE YONG

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF MEDICINE (INTERNAL MEDICINE)

UNIVERSITI SAINS MALAYSIA

2017

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my deepest appreciation my supervisor as well as my lecturer, Assoc. Prof Dr. Azlan Husin for his invaluable suggestions and input, his tireless instructions and teachings as well as for his continuous encouragement. Nevertheless, his role as the Consultant Haematologist has been continuingly providing me with invaluable support and your support was essential to my success here.

My special thanks to my co-supervisors, Dr. Faezahtul Arbaeyah Hussain, Consultant Pathologist who provide indispensable advice, information, and support on a different aspect of my dissertation.

My gratitude also goes to Dr. Erica Kueh Yee Cheng for her exceptional teachings and shedding lights into the world of medical statistics.

Last but not least, many heartfelt thanks to all who were involved in this study.

Dr.Teh Chee Yong

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BM	Bone Marrow
CNS	Central Nervous System
CR	Complete Remission
CSF	Cerebrospinal Fluid
DLBCL	Diffuse Large B Cell Lymphoma
ECOG	Eastern Cooperative Oncology Group
IHC	Immunohistochemistry
IPI	International Prognostic Index
LDH	Lactate Dehydrogenase
MRI	Magnetic Resonance Imaging
PET-CT	Positron Emission Tomography – Computed Tomography
PR	Partial Response
RCHOP	Rituximab, Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone

USM Universiti Sains Malaysia

SPSS Statistical Package for the Social Science

ABSTRAK

Prevalens Expresi Berganda Di Kalangan Pesakit Dewasa DLBCL Dan Factor –Factor Yang Berkaitan Dengannya Berserta Kesan Rawatan Di Hospital Universiti Sains Malaysia Dari Tahun 2004 Sehingga 2015.

Latar belakang dan Objektif:

Diffuse Large B-Cell Lymphoma (DLBCL) merupakan penyakit yang kompleks. Rawatan piawai (RCHOP) gagal memberi kesan rawatan yang optimum justeru menyumbang kepada prognosis yang teruk. Tujuan kajian ini adalah untuk menentukan prevalens Expresi Berganda di kalangan pesakit dewasa DLBCL dan juga bagi menentukan factor –factor yang berkaitan dengannya, di samping kesan rawatannya terhadap rawatan piawai.

Metod:

Kami melakukan kajian rekod retrospektif yang melibatkan pesakit DLBCL dewasa 2004-2015 di Hospital Universiti Sains Malaysia. Maklumat demografi, klinikal dan makmal diekstrak daripada rekod perubatan, manakala status ekspresi berganda diekstrak daripada pangkalan data jabatan histopatologi.

Keputusan:

Sejumlah 27 pesakit DLBCL telah direkrut dalam kajian ini. Prevalensi Ekspresi berganda adalah 59.3%. Ia tiada kaitan signifikan dengan kesan rawatan awal semasa penilaian interim (p =

0.182), tetapi berkait secara signifikan dengan kadar kesembuhan yang rendah terhadap rejimen RCHOP (p = 0.002). Expresi Berganda hanya berkaitan secara signifikan dengan tahap LDH (p = 0.015). Tiada kaitan yang signifikan antara umur, jantina, etnik, peringkat penyakit, gejala B, status ECOG, IPI tersemak, nodal, penglibatan sumsum tulang dan saraf dengan Fenotip Expresi Berganda.

Kesimpulan:

Prevalens Expresi berganda adalah 59.3%. Pesakit dengan Fenotip Ekspresi Double dikaitkan dengan peluang yang lebih rendah untuk mencapai Respon Lengkap dengan rejimen RCHOP. Di antara pemboleh ubah yang dipilih, tahap LDH yang tinggi kekal sebagai faktor penting yang berkaitan dengan Fenotip Ekspresi Double

ABSTRACT

THE PERIOD PREVALENCE OF DOUBLE EXPRESSION DIFFUSE LARGE B-CELL LYMPHOMA PATIENTS AND ITS ASSOCIATED RISK FACTORS AND TREATMENT RESPONSE IN HOSPITAL UNIVERSITI SAINS MALAYSIA FROM YEAR 2004 TO 2015.

Background and Objective:

Diffuse Large B-Cell Lymphoma (DLBCL) is a heterogeneous and complex disease. Its protein expression of MYC and BCL2 and/or BLC6 (Double Expression) is associated with poor response to standard treatment, thus poor prognosis. The aim is to determine the period prevalence of Double Expression phenotype in DLBCL patients and also its associated risk factors and treatment response

Methods:

We performed a retrospective record review involving adult DLBCL patients from 2004 to 2015 in Hospital Universiti Sains Malaysia. Demographic, clinical and laboratory information were extracted from medical record, whereas double expression status was extracted from histopathology department database.

Results:

A total of 27 DLBCL patients were recruited in this study. The period prevalence of Double Expression Phenotypes was 59.3%. It was not significantly associated with early response during interim assessment (p=0.182); but was significantly associated with non-complete response

rate toward RCHOP regimen (p=0.002). It only significantly associated with serum LDH level (p=0.015). There was no significant association between age, gender, ethnicity, stages, B symptoms, ECOG status, revised IPI, Extra-nodal, CNS and bone marrow involvement with Double Expression phenotype.

Conclusion:

The period prevalence of Double Expression Phenotypes was 59.3%. Patients with Double Expression Phenotypes were associated with lower chance to achieve Complete Response with RCHOP regimen. Among the selected variables, raised serum LDH remained the significant factor associated with Double Expression Phenotypes.

CHAPTER 1: INTRODUCTION

1.1 Diffuse Large B-Cell Lymphoma (DLBCL)

DLBCL is the commonest form of Non Hodgkin Lymphoma (NHL), comprising 30-40% of total NHL cases (WHO, 2008). Recent advancement of genetic analysis had revealed its heterogeneity and complexity. Based on cell of origin (COO) classification, DLBCL can be divided into germinal center B-cell (GCB) subtype and activated B-cell (ABC) subtype. Each subtype has different pathology, prognosis, and treatment plan.

DLBCL has an aggressive course of disease progression, usually evolves within months. Without treatment, it may lead to symptomatic diseases and death. It may present with swelling of lymph nodes (either centrally or periphery) and B symptoms, which consist of loss of weight of 10% in 6 months, fever and night sweat. Other symptoms depend on site of lymphoma.

DLBCL is diagnosed via excisional or incisional biopsy of tissues. Biopsy obtained is sent for immune-phenotyping and cytogenetic analysis. Cytogenetic Fluorescence In Situ Hybridization (FISH) studies enable us to detect genetic rearrangement of DLBCL with MYC plus BCL2 or BCL 6, also called as Double Hit Lymphoma.

Double Hit means presence of concurrent MYC and BCL2/6 gene rearrangement, whereas Double Expression means over expression of MYC and BCL2/6 detected via immunohistochemistry staining (Phan RT, 2004).

Double Hit usually belongs to GCB subtype whereas Double Expression is seen in both ABC and GCB subtypes. Both have similar presenting characteristics, associated with advanced stage, extra-nodal disease, poor performance status and high International Prognostic Index (IPI). These characteristics lead to poor prognosis to standard treatment (Allison Rosenthal, 2016).

The most commonly used initial therapy is rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone(R-CHOP). Despite major advances in the treatment, particularly with the addition of the anti-CD20 monoclonal antibody (Rituximab), 40% of the patients still experience early treatment failure after initial response to chemotherapy (Catherine T, 2013).

1.2 Double Expresser Phenotypes

DLBCL may harbor some chromosomal breakpoint affecting the MYC/8q24 locus in combination with other breakpoint which are BCL2/t(14;18)(q32;q21) and BCL6 (Kieron D, 2014). MYC expression is associated with uncontrolled cell growth, division, and metastasis (Adhikary S, 2005). BCL2 is an anti-apoptotic gene which

may extend cell survival (Korsmeyer SJ, 1992). BCL6 encodes a transcriptional repressor and when overexpressed can down regulate several genes, which may subsequently allow DNA-damaged cells to escape from apoptosis (Phan RT, 2004).

Over the past few years, several studies have identified that a significant proportion of DLBCL cases have high protein expression of MYC and BCL2, but frequently without translocations via immunohistochemistry (Johnson NA, 2012). It is recognized that MYC, BCL2 and BCL6 can be activated through various mechanisms, leading to high expression of these protein products (Kieron D, 2014). Recent studies identified 20-40% of DLBCL patients have high MYC and BCL2/6 protein co-expression, detected by IHC (Perry AM, 2014).

High MYC/BCL2 co-expression was associated with an aggressive clinical course and inferior outcome after standard RCHOP chemotherapy (Shimin Hu, 2013). Median survival are often in months, with most reported to be no more than 1.5 years and with overall survivals at 3 or 5 years shorter than expected with usual DLBCL (Pillai RK, 2013).

1.3 Protein Expression Detection

There are many methods used for protein detection, e.g. Enzyme-linked Immunosorbent Assay (ELISA), Gel Electrophoresis, Western blot, Immunoprecipitation, Spectrophotometry, Immunohistochemistry and etc.

Immunohistochemistry method was used for MYC, BCL2 and BCL6 protein detection in Histopathology Department Database under the Study "The Immunohistochemical Profile of Double-Hit Diffuse Large B-Cell Lymphoma and Their Association with Clinicopathological Parameters: A Pilot Study in Hospital" by WN Najmiyah et al (2016). Double Expression status in this study was extracted from this database.

1.3.1 Immunohistochemistry

IHC is used to detect Lymphoma protein expression via primary antibody (monoclonal antibody) staining.

1.3.1.1 Tissue Processing

The selected single representative block for each case was sectioned to 3-micron thickness and mounted on commercially available poly-L-lysine pre-coated slides. All cases were stained with H&E, re-evaluated their histopathological diagnosis and ensure adequate tumor tissues were present for immunohistochemistry staining.

1.3.1.2 Primary Antibodies

BCL6 primary antibody that was used in this study is clone PG-B6P, whereas BCL 2 primary antibody that was used in this study is clone 124. Both were manufactured and marketed by DAKO Denmark A/S. These antibodies were monoclonal antibody that derived from a mouse. Human tonsil was used as positive control. Anti-c-MYC primary antibody that was used in this study was clone Y69 (catalog No. AB32072)

manufactured and marketed by Abcam, Cambridge, MA. This antibody derived from rabbit. Human colonic adenocarcinoma tissue was used as positive control.

1.3.1.3 Immunohistochemical Staining Method

Each antigen had a preferred method of antigen retrieval, and each antibody had an optimal dilution. These optimizations include antigen retrieval, primary antibody concentration and detection. After optimization, the procedures of IHC staining for BCL2 and MYC (except BCL 6) were described below:

a) Sectioning

The selected tissue paraffin blocks were sectioned at 3 microns using microtome. The ribbons of the sections were floated in water-bath at temperature of 50°C. Then they were fished and mounted onto the poly-L-lysine treated glass slide. The slides were allowed to dry on the slide-drying stand at room temperature.

b) Deparaffinization and hydration

The slides then were placed on a hot-plate at temperature of 60°C for an hour. These steps were important for tissue adherence and to avoid peeling. Hydration process began with immersion into Xylene I and II at 5 minutes each. This followed by immersion into 100% concentrated alcohol twice at 2 minutes each. Subsequently, they are immersed into alcohol with reducing concentration from 95%, 80%, 70% to 50%. Each of the processes was done at 2 minutes intervals. The slides then were rinsed in distilled water.

c) Antigen retrieval

Buffer solution used for heat induced-epitope retrieval (HIER) in BCL2 and MYC was Tris buffer, 1mmol/L EDTA, pH9.0. The antigen retrieval was done using pressure cooker method. Once the buffer boils, the slides were transferred into the pressure cooker and heated for 3 minutes. The pressure cooker then was cooled under running water for 15 minutes followed this. The slides were then rinsed using distilled water for 5 minutes.

d) Endogenous peroxidase blocking

The slides were soaked with 3% Hydrogen peroxidase (H_2O_2) for 5 minutes then rinsed with Tween 20 buffer. This step allowed the endogenous peroxidase activity to neutral and reduced the background staining.

e) Incubation with primary antibody

BCL2 and MYC primary antibody at dilution of 1:50, 1:50, 1:50, 1:500 and 1:50 respectively were applied to the sections and incubated for 1 hour at room temperature. This was followed by rinsing twice using TrisBuffer Saline (TBS).

f) Incubation with Horseradish peroxidase (HRP) polymer solution

Two drops of the HRP secondary antibody were applied onto the sections and were incubated for an hour at room temperature. Sections were then rinsed twice using TBS.

g) Chromogen application

One drop of 3, 3'-diaminobenzidine (DAB) from DAKO chromogen was applied to the sections and incubated for a minute at a room temperature. The sections were then rinsed with distilled water.

h) Counterstaining

The sections were counter-stained with haematoxylin for 10 seconds and washed in running water for 3 minutes before commencing the hydration processes.

i) Dehydration

This process was the reverse process of hydration. The sections were immersed in alcohol with gradual increased in concentration, from 70% to 80%, twice in 95% and twice in 100% at 1-minute interval each. This was followed by dipping into 2 changes of xylene at 2 minutes each.

j) Mounting

The final process was mounting the tissue section using Cytoseal and protect with cover slips.

The procedure of IHC staining for BCL 6 is as described below:

a) Sectioning

The selected tissue paraffin blocks were sectioned at 3 microns using microtome. The ribbons of the sections were floated in water-bath at temperature of 50°C.

Then they were fished and mounted onto the poly-L-lysine trated glass slide. The slides were allowed to dry on the slide-drying stand at room temperature.

b) Deparaffinization and hydration

These slides were placed on a hot-plate at temperature of 60°C for an hour. Hydration process began with immersion into Xylene I and II at 5 minutes each. This followed by immersion into 100% concentration of alcohol twice at 2 minutes each. Subsequently, immersed into alcohol with reducing concentration from 95%, 80%, 70% to 50%. All these processes were done at 2 minutes intervals. The slides were then rinsed in distilled water.

c) Antigen retrieval

Buffer solution used for heat induced-epitope retrieval (HIER) in BCL6 is Tris buffer, 1mmol/L EDTA, pH9.0. The antigen retrieval was done using pressure cooker and was put on hotplate. Once the buffer boiling, the slides were transferred inside the pressure cooker and cook for 3 minutes. This was followed by cooling for 20 minutes. When cooled, the slides placed in TBS with 0.05% Tween 20.

d) Incubation with primary antibody

BCL6 primary antibody at dilution of 1:40 was applied to the sections and incubated for 30 minutes at room temperature. Sections were rinsed twice using TrisBuffer Saline (TBS).

e) Endogenous peroxidase blocking

These slides were soaked with 3% Hydrogen peroxidase (H_2O_2) for 5 minutes and rinsed with Tween 20 buffer.

f) Incubation with DAKO Mouse Linker

DAKO Mouse Linker was applied to the sections and incubated for 20 minutes. The sections were rinsed twice using TrisBuffer Saline (TBS).

g) Incubation with Horseradish peroxidase (HRP) polymer solution

Two drops of HRP secondary antibody were applied onto the sections and incubated for an hour at room temperature. Sections were rinsed twice using TBS later.

h) Chromogen application

One drop of DAB or 3, 3'-diaminobenzidine from DAKO chromogen was applied to the sections and incubated for a minute at a room temperature. These sections were rinsed with distilled water later.

i) Counterstaining

The sections were counter-stained with haematoxylin for 10 seconds and washed in running water for 3 minutes before the dehydration processes.

j) Dehydration

The sections were immersed in alcohol with gradual increased in concentration, from 70% to 80%, twice in 95% and twice in 100% at 1-minute interval each. This was followed by dipping into 2 changes of xylene at 2 minutes each.

k) Mounting

The final process was mounting the tissue section using Cytoseal and protect with cover slips.

1.3.1.4 Interpretation

The staining pattern for MYC protein and BCL6 was distinctly nuclear, whereas staining BCL2 protein showed a well-defined cytoplasmic staining pattern. In this study, the cut point of dichotomizing expression of MYC and BCL2 were ≥40% and ≥50%, respectively in accordance to the previously established cutoff points (Johnson *et al.*, 2012; Savage *et al.*, 2016). The cut point of dichotomizing expression of BCL6 was >50% based on previously established cutoff point (Ye *et al.*, 2016). These cases are classified as double expression if it has MYC/BCL2 or MYC/BCL6.

1.4 International Prognostic Index (IPI)

International Prognostic Index Study was published in year 1993 (James OA, 2005). This study showed 5 factors with roughly equal power in predicting treatment outcome and prognosis. These are:

1. Age > 60 years
2. Ann Arbor stage III/IV
3. More than 1 extra-nodal site
4. Serum LDH above normal
5. ECOG performance status ≥2

With that, patients may be divided into 4 groups of low risk (score of 0-1), low intermediate risk (score of 2), high intermediate risk (score of 3) and high risk (score of 4-5). Thus predicting the prognosis in term of 5 years overall survival and complete remission rate (James OA, 2005).

However, with the introduction of Rituximab in Standard Chemotherapy regimen, it had significantly improved the survival of DLBCL patients, thus altered previously understanding and prediction of clinical outcome. This leads to revision of IPI (Revised IPI). It identified 3 different prognostic groups and provided a better prediction of clinical outcome. Patients with zero factors have very good prognosis and more than 2 factors have poor prognosis (Laurie HS, 2007).

Risk group	No. of IPI factors	% Patients	4-year PFS, %	4-year OS, %
Standard IPI				
Low	0, 1	28	85	82
Low-intermediate	2	27	80	81
High-intermediate	3	21	57	49
High	4, 5	24	51	59
Revised IPI				
Very good	0	10	94	94
Good	1, 2	45	80	79
Poor	3, 4, 5	45	53	55

Table 1.1 International Prognostic Index Interpretations

Shimin Hu et al (2013) in their study showed DLBCL with Double Expression had clinicopathological features associated with aggressive clinical course. This group of patients

had significant extra nodal involvement, higher serum LDH level, and thus higher IPI score in comparison with non-Double Expression, which may translate into poorer adverse outcome and thus prognosis. However, B symptoms were not significantly rose in Double Expression group.

1.5 Ann Arbor Staging System

Ann Arbor Staging originally was developed for Hodgkin disease, but it serves as a basis for anatomic staging in non-Hodgkin Lymphomas as well (James OA, 2005). It helps clinician to stage the disease and determine the treatment plan in a more rational and objective way (WHO, 2008).

This system divides patients into 4 stages based on localized disease, multiple site disease on one side of diaphragm, lymphatic disease on both sides of diaphragm and disseminated disease. Localized extra-nodal sites and spleen involvement are recognized by a subscript E and S respectively (James OA, 2005). The definition of these stages listed in the table below.

Stage	Features
I	Involvement of a single lymph node region or lymphoid structure (eg, spleen, thymus, Waldeyer's ring)
II	Involvement of two or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph regions or structures on both sides of the diaphragm
IV	Involvement of extranodal site(s) beyond that designated E
For all stages	
A	No symptoms
B	Fever (>38ºC), drenching sweats, weight loss (10% body weight over 6 months)
For Stages I to III	
E	Involvement of a single, extranodal site contiguous or proximal to known nodal site

Table 1.2 Ann Arbor Stages and Features

Staging in non-Hodgkin Lymphoma can be done using history, physical examination, laboratory studies, and images (Computerized Tomography Scan and Magnetic Resonance Imaging), in addition to definitive proof of involvement of a particular site from biopsy.

As mentioned at previously, DLBCL with double expression is associated with aggressive disease progression thus advanced stage of disease. This is true as mentioned by Shimin Hu et al (2013) in their study, which showed that Double Expression group had significantly higher number of patients diagnosed with Stage III and IV.

1.6 Eastern Cooperative Oncology Group (ECOG) Score (Oken M, 1982)

This is a score that attempts to sort patients to variable group based on their general well-being, daily activity and performance status. It serves in a more objective way, to quantify the quality of life of these patients.

Grade	Description
0	Fully active, able to carry on all predisease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature (eg, light housework, office work)
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

Table 1.3 ECOG Grade and Description

Performance status of a patient is not solely based on the aggressiveness and stages of the disease. Study by Shimin Hu et al (2013) showed that ECOG status is poorly associated with Double Expression status.

1.7 Bone Marrow and CNS Infiltration

Bone marrow aspiration and Trephine biopsy examination is vital in evaluation and staging of DLBCL at the time of diagnosis and after therapy. Iliac crest is considered the best spot for bone marrow biopsy. Biopsy specimens were studied for cellularity, hematological elements, extent of infiltration, histologic pattern and morphology of infiltration, reticulin fibrosis and other secondary changes (Suneet K, 2009).

Central nervous system involvement in DLBCL is diagnosed by patients' history, clinical examination, Imaging (CT scan and MRI) and CSF examination via lumbar puncture. CNS involvement is considered when there is meningeal infiltration showed in imaging or CSF showed positive cytology result (A Hollender, 2002).

Both CNS and BM involvement is associated with poor prognosis. As DLBCL with Double expression is associated with advance and aggressive behavior of disease, one would expect the likely hood of involvement in this group. Literature review showed no available data at the time of study addressing these.

1.8 Treatment Response

The standard chemotherapy regimen for DLBCL is RCHOP, which consists of Rituximab, Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone. The

regimen is given for 6 cycles with 21 days interval in between cycle. Interim assessments in the form of clinical examination and imaging studies (CT scan) are undertaken to decide for treatment responses. Cheson Criteria was used to define the following treatment response (Cheson BD, 2014):

- Complete Remission (CR) is the disappearance of all evidence of diseases, which include regression of nodal mass to its normal size, regression of hepatosplenomegaly and cleared of bone marrow infiltration during the time of assessment.
- Partial Response (PR) was considered when tumor mass or organ infiltration decreased by at least 50 % along with the disappearance of disease-related symptoms during the time of assessment.

1.8.1 Complete Response

Complete response is defined as patients who achieved complete remission upon completion of 6 cycles of chemotherapy. Shimin Hu et al noted DLBCL with double expression had significantly lower Complete Response rate (Shimin Hu, 2013). Double Expression does not response well with current chemotherapy regimen i.e. RCHOP.

1.8.2 Early Response

Identification of early response status during the interim assessment is of vital important in order to maximize the chances of second line or salvage chemotherapy success, avoid the unnecessary side effect of failed first line chemotherapy and

cutting down cost of treatment. In the Lugano criteria published in 2014 (Cheson BD, 2014), PET-CT is recommended for staging as well as response assessment following therapy, as it is the most accurate imaging modality.

PET-CT emerged as a reliable biomarker for assessing early response in DLBCL. It assesses tumor metabolic activity (via standard uptake volume-SUV), metabolic tumor volume (MTV) and metabolic tumor burden (MTB). Thus give rise to a better accuracy in detecting tumor aggressiveness as to compare to biopsy and may predict early treatment response as early as 1 week after chemotherapy (Xingchen Wu, 2014).

Besides the criteria as per defined based on PET/CT (which is not widely available), there are no standard universal criteria for Early Response. Thus, in this study, patients who achieved complete remission or partial remission during interim assessment are considered as early responder.

Literature review showed no available data at the time of study addressing early response in DLBCL with double expression.

1.9 Rationale of the Study

DLBCL is a heterogeneous and complex disease. Its Double Expression is associated with poor response to standard treatment and thus may translate into poor

outcome/prognosis (Allison Rosenthal, 2016). Multiple studies had concluded that the median survivals of DLBCL with Double Expresser Phenotypes are often in months, with most reported to be no more than 1.5 years and with overall survivals at 3 or 5 years shorter than expected with usual DLBCL (Valera A, 2013).

The DLBCL Double Expression statistic mentioned originate from oversea country, which may be compounded by factors such as ethnic group, lifestyle, dietary, geographic and etc. The prevalence differs among countries: Denmark (29%)(Green TM, 2012), United State (7-34%)(Allison Rosenthal, 2016), Canada (21-44%) (Johnson NA, 2012), Korea (10-16%)(Sehui Kim, 2016), Taiwan (2%)(ST Chang, 2016), Japan (5%)(K Kawamoto, 2016) and etc.

This study aims to produce our own set of statistic (as currently no such available) which may reflect local pictures and raise awareness on these phenotypes. With that we hope all DLBCL patient pathology samples should be tested for MYC, BCL2, and BCL6 expression.

Knowing the aggressiveness of Double Expression DLBCL, this study will try to identify some added associated risk factors of Double Expression Phenotypes, which may lead to prediction of such phenotypes among DLBCL patients. These identifiable associated clinical risk factors may be used as a surrogate marker if were proven to be strongly significant, hence optimizing the additional specific laboratory testing on

diagnostic tissue sample. With early prediction of Double Expression phenotypes, this may facilitate decision-making on best chemotherapy regimen option in the future.

This study also assessed early response via interim CT scan, which is more applicable in most cancer centers in developing countries in which PET-CT are not widely available. Identification of early response status is of vital important in order to maximize the chances of second line or salvage chemotherapy success, avoid unnecessary suboptimal response to ineffective treatment and cutting down treatment cost.

CHAPTER 2: OBJECTIVES

2.1 General Objective

To determine the period prevalence of Double Expression DLBCL patients and its associated factors and treatment response in HUSM from 2004 to 2015.

2.2 Specific Objective

2.2.1 To determine the period prevalence of Double Expression of lymphoma protein MYC and BCL2/6 among Diffuse Large B-Cell Lymphoma patients in HUSM from 2004 to 2015.

2.2.2 To determine the association between treatment response and this Double Expression among DLBCL patients in HUSM from 2004 to 2015?

- Complete Response
- Early Response

2.2.3 To determine the various demographic and clinical factors that may be associated with this Double Expression among Diffuse Large B-Cell Lymphoma patients in HUSM from 2004 to 2015.

- B symptoms
- LDH
- ECOG status

- Stage (Ann Arbor Staging System)
- Extra-nodal Site
- Age
- Gender
- CNS involvement
- BM involvement
- Revised IPI risk group

2.3 Hypothesis

Alternative Hypothesis

There are significant associated risk factors and treatment response among Double Expression DLBCL patients in HUSM from 2004 to 2015.

Null Hypothesis

There are no significant associated risk factors and treatment response among Double Expression DLBCL patients in HUSM from 2004 to 2015.

CHAPTER 3: METHODOLOGY

3.1 Study Design

Retrospective record review.

3.2 Study Population

Patients who were newly diagnosed with DLBCL and treated in HUSM from year 2004 until 2015.

3.3 Study approval

This study was approved by the Research and Ethic Committee, Universiti Sains Malaysia on 23rd March 2017. Study protocol number USM/JEPeM/16120584.

3.4 Inclusion criteria (to all objectives)

1. All patient that was newly diagnosed with DLBCL and treated in Hematology Unit, Hospital Universiti Sains Malaysia.
2. Underwent at least 3 cycle of chemotherapy of RCHOP or R-mini CHOP.
3. The availability of MYC, BCL2 and BCL6 results.

3.5 Exclusion criteria (to all objectives)

1. Transformed DLBCL
2. Relapsed or Progressive DLBCL
3. Patient with other form of malignancy (double pathology)
4. Patient did not receive RCHOP/R- mini CHOP.

3.6 Sample Size Calculation

Objective 1

Sample size is based on prevalence of DLBCL double expression phenotypes

$N = [z / \Delta]^2 p (1 – p)$

P = prevalence of DLBCL Double Expression Phenotypes = 0.3[17-18]

Z = 1.96 for 95% CI

Δ (precision) = 0.1

$N = [1.96 / 0.1]^2 0.30 (1 – 0.30)$

= 81

Missing data estimated to be 10% = 62 x 10% = 90

The sample of 90 will be required at analysis stage.

Objective 2

P_0 is the probability of exposure to variables in DLBCL patients without Double Expression. These Variables were obtained from *Shimin Hu et al* (2013).

P1 is an expert opinion about the probability of exposure to variables in DLBCL patients with Double Expression.

The sample size calculated using Power and Sample Size Calculation version 3.0.10

α = 0.05, power = 0.8, ratio (m) of 1:1; P0 = 0.84; P1 = 0.65, n = 81, Total n (adding 10%) = 180

A sample size of 181 DLBCL patients will be required for this study

Objective 3

P_0 is the probability of exposure to variables in DLBCL patients without Double Expression. These Variables were obtained from *Shimin Hu et al* (2013).

P1 is an expert opinion about the probability of exposure to variables in DLBCL patients with Double Expression.

The samples size calculated using Power and Sample Size Calculations version 3.0.10

α = 0.05; Power = 0.8; ratio (m) of 1:2

VARIABLES	P_0	P_1	N	TOTAL N (Including 10% Missing Data)
B symptoms	0.28	0.5	56	186
LDH	0.58	0.8	52	173
ECOG status	0.10	0.3	44	147
Stage (Ann Arbor Staging System)	0.43	0.7	39	130
Extra-nodal Site	0.18	0.4	47	157
Age (>60) Gender (Male) CNS involvement BM involvement	**There are no available data at the time of study design to estimate sample size based on the listed variables**			
IPI Risk Group	0.30	0.55	44	147

Table 3.1 Sample Size Calculation

A sample size of 186 DLBCL patients will be required for this study, assuming α = 0.05, power = 0.8, ratio (m) of 1:2 and 10% of missing data.

3.7 Patient Recruitment and Sampling Method

Diffuse Large B-Cell Lymphoma patients diagnosed in HUSM from 2004 till 2015 were traced from medical record unit. Total of 278 patients were identified but only 222 patients fulfilled inclusion and exclusion criteria. To be eligible, these subjects must be newly diagnosed with DLBCL and treated in Hematology Unit, Hospital Universiti Sains Malaysia, excluding transformed, relapsed or progressive DLBCL treated